Attorney Docket No.: ANDE-001/04US 307109-2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Norman L. Anderson	Confirmation No.:	6420
Appl. No.:	10/676,005	Examiner:	Jana A. Hines
Filing Date:	October 2, 2003	Art Unit:	1645
Title:	HIGH SENSITIVITY QUANTITATION OF PEPTIDES BY MASS SPECTROMETRY		

DECLARATION OF DR. STEVEN CARR UNDER 37 CFR § 1.132

Declaration under 37 C.F.R. § 1.132

I, Dr. Steven Carr, declare and say:

1. I am Director of Proteomics and Biomarker Discovery at the Broad Institute of Harvard and MIT, Cambridge Massachusetts.

2. A copy of my *Curriculum Vitae* is appended below as APPENDIX A.

3. I am the Principal Investigator on a $15M 5 year grant from the National Cancer Institute aimed at implementing SISCAPA assays of candidate cancer biomarkers. I am also a participant in discussions regarding commercialization of the SISCAPA technology.

3. A primary goal of proteomic technology since its inception in the mid-1970's has been to observe and detect changes in as many proteins as possible in complex biological samples. Early proteomic methodology used 2-D gels, but these were difficult to run, had difficulty in providing reproducible quantitative data, and had limited dynamic range.

4. In the 1990's mass spectrometric ("MS") methods emerged, usually based on the analysis of 5-25 amino acid peptides in mass spectrometers. In the 'shotgun proteomics' method various fractionation schemes were used to divide a complex protein digest into fractions compatible with the resolving power, dynamic range and throughput of available mass spectrometers. As potentially useful biomarkers began to emerge from this research, it became apparent in 2000-2001 that accurate, sensitive quantitation was necessary to further investigate biomarker candidates.

5. Some investigators pursued a strategy of adding a quantitative capability to existing shotgun proteomics approaches, for example by labeling peptides prior to MS detection. By adding different labels to different samples, the labeled samples could be mixed, resolved and measured in the mass spectrometer, which could compare the relative amounts of each peptide in its various labeled and unlabeled forms. This method still required sample fractionation to divide the sample into relatively simple subsets of

peptides. As a result, the time and effort required per sample proved to be prohibitive for measuring proteins in the large (1,000-10,000) samples sets typically required to assess candidate biomarkers for clinical use. As a consequence, not a single biomarker discovered using proteomics has yet entered widespread clinical use.

6. A potential alternative method for assessing biomarker candidates available in 2002, was to use quantitative triple-quadrupole MS operated in multiple reaction monitoring ("MRM") mode) using isotope dilution assays. This method had been used for quantitative analyses of small molecule drugs and their metabolites extracted from complex mixtures such as plasma. In this MRM method, isotope-labeled versions of the analyte of interest were used as internal standards, which were spiked into a sample prior to analysis to allow accurate quantitation of the analyte by ratio with the standard.

7. Small molecules and their metabolites typically can be isolated from a complex sample by routine extraction with organic solvents or by a simple solid-phase extraction method. This provides a relatively uncomplicated mixture for MRM MS analysis.

8. By stark contrast, the problem with using this MRM MS technique to study protein biomarkers is not only the vastly increased variety of peptides in a complex sample digest but the wide range of relative abundance of the peptides. Later research has confirmed that in unfractionated samples the MRM technique is able to measure peptides derived only from about 50 of the most abundant plasma proteins. This therefore precludes measuring most known biomarkers and, more significantly, biomarker candidates.

9. Some investigators tried to fractionate samples using class-specific affinity reagents such as lectins, while others used strong cation exchange or size exclusion chromatography. These added fractionations typically yielded very modest enrichments of 5-20-fold, and required lengthy and tedious analysis of the multiple fractions thus generated.

10. By 2002 there was some evidence that a few antibodies could bind large peptides containing specific epitopes and, by binding, enrich these peptides with respect to other peptides from the same protein in a process called "epitope mapping". The purpose of this approach was to identify the antibody binding site on the protein. The Zhao paper describes a process in which one or a few peptides are enriched from the handful of peptides (e.g., 5-20) observed by MALDI MS in a simple digest of one protein. The epitope mapping approach did not involve (and did not require) any quantitative assessment of the enrichment, since its object was qualitative structural information; i.e. which peptide binds to the Ab (at all) and which others do not. For epitope mapping, a 10-fold enrichment of a single peptide from a mixture of about 10 peptides is sufficient.

11. A digest of one or two proteins is orders of magnitude less complex than a digest of a complex biological sample such as plasma, for at least two reasons: First, plasma contains at least 1,000 different proteins at concentrations above the pg/ml level. Since each protein produces on average 50 tryptic peptides (based roughly on the molecular weight of the protein), a plasma digest is thus expected to contain > 50 x 1,000 = 50,000 peptides. The number represents ~5,000 to 10,000-fold more peptides than the handful of peptides that were observed in epitope mapping experiments.

12. Second, the peptides produced by digesting a single protein (such as those obtained in an epitope mapping experiment such as that described by Zhao) are present in the digest in more or less equal amounts. By contrast, in a complex sample such as plasma, there are enormous differences in concentration between one protein and another, and this very wide difference in concentration results in enormous differences in concentration of individual peptides in a digest prepared from the complex sample. It is widely believed that there are more than 10e10 (i.e., 10,000,000,000) – fold differences between the amounts of specific proteins in plasma (e.g., albumin and IL-6).

13. The methods described in the claims of the captioned application ("SISCAPA") can be used to enrich peptides from low abundance proteins, raising them to a level that is detectable and measurable by MS. Accordingly, SISCAPA requires

capturing a peptide from a lower-abundance protein in the presence of a great many more copies of peptides from higher abundance proteins. The initial target for SISCAPA sensitivity is in the range between 1ng/ml to 1μg/ml in plasma, whereas albumin and other high abundance proteins are present at 10-50 mg/ml. These levels are 10,000 to 50,000,000 fold higher than those of a SISCAPA target protein. After digestion of plasma to peptides, the digest will contain 10,000 to 50,000,000 times as many molecules of peptides from the high abundance plasma proteins as from the target protein.

14. These very large differences in complexity mean that the target peptide to be enriched by an antibody in the SISCAPA method is likely to be 1 of more than 50,000 peptides in a plasma digest. Moreover, many of these 50,000 or more other peptides will be present in concentrations that are 10,000 to 50,000,000-fold higher than the peptide of interest. A reasonable calculation therefore would suggest that for a typical protein target of a SISCAPA assay, present at 10ng/ml in plasma, there are likely to be 200,000,000 molecules of competing peptide (including 87,000,000 molecules of albumin peptides) for each molecule of the desired peptide derived from the target protein. Thus huge enrichment factors would be required to enrich and 'isolate' the desired peptide.

15. The prejudice in the field in 2002 was that it was not possible to use an antibody to substantially enrich a short tryptic peptide from a complex matrix such as that produced by digesting a bodily fluid, such as plasma. The references cited by the Examiner describe epitope mapping experiments using single protein digests, where the digest produces maybe 50 peptides, present at essentially equal concentration. By comparison, the complex protein digests obtained from bodily fluids contain 5,000-10,000 times the number of different peptides, and this much great variety of peptides is also present in a concentration range that varies 10,000 to 50,000,000-fold between peptides. At the time the captioned application was filed, there was no expectation that antibodies (or any other specific binding agent) could be used to select one peptide from a complex plasma digest.

16. It was therefore extremely surprising to me and to other workers in the field when it was shown that the methods described in the captioned application were

able to provide substantial enrichment of target peptides from complex digests of bodily fluids, and that the methods could be used to study proteins that are present at low concentration (low ng/ml) in these fluids.

17. All statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any document or any registration resulting therefrom.

Date: 23 - AUGUST - 2008

Dr. Steven Carr